Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose–response curves

DE LEAN, A., P. J. MUNSON, AND D. RODBARD. *Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves.* Am. J. Physiol. 235(2): E97–E102, 1978 or Am. J. Physiol.: Endocrinol. Metab. Gastrointest. Physiol. 4(2): E97–E102, 1978.—Physiological and pharmacological studies of hormones, drugs, and neurotransmitters often generate families of sigmoidal dose–response curves. Optimally efficient data analysis should involve simultaneous description of all curves, rather than fitting each one individually. We have developed a general computerized method to describe the dose–response curves in terms of basal and maximal responses, ED_{50}, and curve shape or steepness. This facile method permits rigorous statistical analysis, provides a basis for pooling of information from separate experiments, and allows one to test which characteristics are shared by various curves.

data analysis; curve fitting; hormone receptors; radioimmunoassay; neurotransmitters, DNA-RNA hybridization

DOSE–RESPONSE CURVES from bioassays, radioreceptor assays, radioimmunoassays (RIA), and DNA–RNA hybridization are typically smooth, symmetrical, and sigmoidal (S-shaped) when the dose is portrayed on a logarithmic scale. Usually, these curves may be equally well described by the Gaussian cumulative distribution (probit analysis) or by a logistic model (15, 29). The latter has advantages of mathematical simplicity and has been widely used for bioassay, radioimmunoassay, and related techniques (2, 4, 5, 7, 10, 12–14, 20, 27–29, 31, 33, 35–37). The general form of the logistic function may be expressed as

$$Y = \frac{a - d}{1 + (X/c)^b} + d$$

where Y is the response; X, the arithmetic dose; a, the response when $X = 0$; d, the response for "infinite" dose;[1] c is the ED_{50}, i.e., the dose resulting in a response halfway between a and d; and b is a "slope factor" that determines the steepness of the curve.[2]

This equation has been used as the basis for analysis of dose–response curves, individually. When two or more dose–response curves have been constructed, the usual practice has been to characterize each one separately and then to compare the slopes and potencies, e.g., in terms of the ratios of the ED_{50}'s. However, this

$$dY/d\ln(X) = (d - a)b/4 \qquad \text{when } X = c$$

or

$$d\ \text{logit}\{(Y - d)/(a - d)\}/d\ln(X) = \pm b$$

The logistic equation is mathematically analogous to the Hill equation used for ligand binding and enzyme kinetics. The parameter b has the same mathematical form as the Hill coefficient, n_H. However, b cannot be interpreted in the same thermodynamic terms as n_H except under very special circumstances. In the proper application of the Hill equation, X refers to free ligand concentration, whereas in most applications of the logistic equation (including those in this report), X indicates total ligand concentration (29). The finding of a b value greater than unity may indicate positive cooperativity or a host of other interpretations. For instance, the presence of a significant threshold and spare receptors will elevate the b value (24). Whether true cooperativity is involved is a moot point. In another commonly occurring case, heterogeneity of binding sites is reflected in an n_H of less than unity. Usually (though not always), the b value measured when $Y = (a + d)/2$ will also be less than unity. However, extensive simulation studies have indicated that the b value may be far removed from the true n_H (unpublished observations). Hence, slopes of logit–log plots of RIA's and RRA's should not be referred to, nor interpreted as Hill coefficients. Instead, these should be simply referred to as b values, or logit–log slopes.

[1] Reversal of the roles of a and d reverses the sign of b. The parameters a and d may be alternatively defined as the minimum and the maximum of the response range, respectively. With the initial definition (responses at zero and infinite dose, respectively), b is always positive. In the alternative definition, the sign of b depends on whether the response is increasing (b positive) or decreasing (b negative) with increasing dose X.

[2] The slope factor, b, corresponds to the slope of the logit–log plot, when X is portrayed in terms of natural logarithms

approach does not extract all of the "information" from the experimental data: to do so, it is necessary to analyze all of the curves simultaneously, forcing them to share certain parameters in common, if warranted by a priori considerations and by the data (28, 38, 39). This situation commonly arises in structure–function studies of hormones, drugs, neurotransmitters: indeed, whenever a series of chemical analogues are assayed simultaneously. In such cases, we may expect all analogues to share some parameters (e.g., the basal response, a); some analogues may share additional parameters (e.g., all full agonists will have the same maximal responses, d), whereas other subgroups of compounds may share additional parameters (e.g., all antagonists might have the same slope factor, b). In general, the parameter c will differ for each dose-response curve.

Use of "constrained" curve fitting not only provides more information; it may also be essential in order to permit the curve-fitting routine to provide reasonable estimates of parameters. Some of the curves may fail to provide any information about one or more of the parameters of the logistic equation if the curves do not span the full range of responses. For instance, some curves (for very weak agonists) may provide information regarding a, but none regarding b, c, or d. Similarly, for very potent preparations (e.g., superagonists), we may attain a maximal response at the lowest dose tested in a particular assay. This would provide information solely about d. Other preparations, assayed in the vicinity of their ED_{50}'s, may provide information regarding b and c, but not a or d.

In the present report, we describe the development and application of a computer program for simultaneous curve fitting of families of dose–response curves based on the four-parameter logistic equation, which permits us to constrain the solution so that curves share desired common parameters. This program, written in extended BASIC, is readily adaptable to minicomputers and desk-top calculators, does not require the large scale computer facilities necessary for currently available general purpose modeling packages (3, 21, 34) and is intended for the biochemical laboratory performing bioassays and radioligand assays. The program provides automated statistical analyses to evaluate "goodness of fit" and to test the hypothesis that certain curves share selected parameters in common. We shall briefly describe the mathematical and statistical bases for this program and illustrate its use with several representative examples.

METHODS

We employ a general nonlinear, least-squares curve-fitting routine[3] using the Gauss-Newton algorithm as modified by Marquardt and Levenberg (18, 22). Selection of shared parameters is made interactively[4] and the general curve fitting model is automatically modified to accommodate the constraints. In cases in which there is nonuniformity of variance, the program permits use of weighting, by use of either a linear, parabolic, or power-function relationship between the variance (σ^2) of Y and the Y level.[5,6] Goodness of fit is evaluated on the basis of the residual variance, by use of the "extra sum of squares principle," which is only approximate when applied to nonlinear models (11). Any constraint (parameter sharing) will increase the sum of squares of the residuals but will also decrease the effective number of parameters estimated. If the gain in the number of degrees of freedom (number of data points minus number of estimated parameters) counterbalances the gain in the sum of squares of residuals, the F test will be small (around 1), indicating the appropriateness of the constraints used. Randomness of the residuals (deviations of observed from predicted responses) is tested by evaluation of the number of "runs" of positive or negative residuals (1, 11). The data points are expected to be randomly distributed above and below the fitted curve if the model is appropriate. Significant nonrandomness of the signs of the residuals indicates an inappropriate fit.

EXAMPLES

We shall illustrate the utility and versatility of this approach to data analysis, by means of four examples: *1*) RIA estimation of relative potency, in this case of an iodinated antigen, to obtain a measure of specific activity; *2*) comparison of agonists and antagonists in a neurotransmitter radio-receptor assay system; *3*) in vitro bioassay of human chorionic gonadotrophin (hCG) and several of its deglycosylated derivatives; *4*) DNA-RNA hybridization analysis.

1) Radioimmunoassay potency estimation. A simple

[3] For purposes of curve fitting, we reparameterize the logistic equation to utilize $c' = \ln(c)$ (cf. Appendix 3 of ref. 28). This prevents c from becoming negative, widens the range of convergence, and exploits the nearly Gaussian (or at least symmetrical) error distribution for c'. Further, this simplifies calculation of standard errors and confidence limits for the relative potency (17) of two preparations.

[4] The number of parameters estimated from a family of curves constrained to have shared parameters will be less than the total number of parameters if each curve were considered separately. This increases the number of degrees of freedom (number of observations minus number of parameters) for the residual variance and narrows the confidence limits for parameters (e.g., potency estimates), provided that the underlying model is correct.

[5] We utilize weights which are inversely proportional to the predicted variance of the response, Y

$$w = 1/\hat{\sigma}_Y^2$$

where

$$\hat{\sigma}_Y^2 = a_0 + a_1 Y + a_2 Y^2 + a_3 Y^{a_4}$$

The weighting coefficients (a_0–a_4) are estimated in a preliminary analysis of replicates, as described (30).

[6] Commonly, the "within-dose" variance is significantly smaller than the "between-dose" variance around the regression. Accordingly, in general we advise use of the means of replicates as input for the regression program, provided sufficient data are available to ensure convergence. Otherwise, a biased underestimate of the residual variance may be obtained and statistics for selecting among competing models may be biased. When within-dose and between-dose variances are comparable, then each observation should be entered.

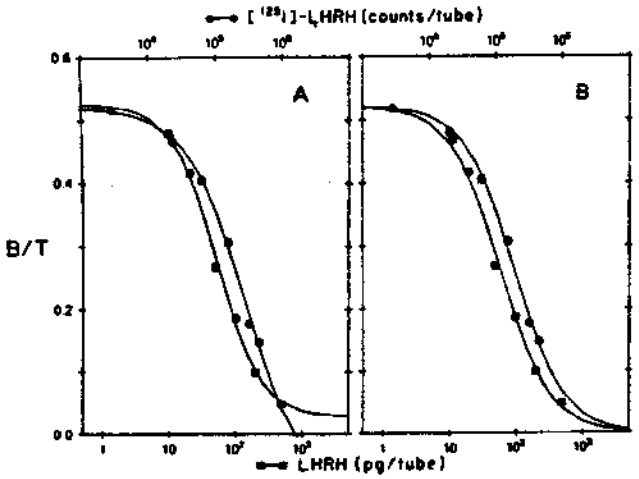

FIG. 1. RIA dose-response curves for native LHRH and ^{125}I-labeled LHRH using high affinity antibodies ($K = 1.5 \times 10^{10}$ M^{-1}). Results are expressed as bound/total ratio for labeled ligand (corrected for nonspecific binding) versus amount of unlabeled hormone (■, lower horizontal scale) or amount of labeled hormone in excess of constant mass of tracer (●, upper horizontal scale). Curves are fitted either independently (*A*) or subject to constraints: $a_1 = a_2 = 0$, $b_1 = b_2$, $d_1 = d_2$ (*B*). Unpublished data, Jean Cote.

example of simultaneous fitting of two sigmoidal curves is shown in Fig. 1, *A* and *B*. The potency estimate[7] of labeled luteinizing hormone releasing hormone (^{125}I-labeled LHRH) relative to native LHRH was measured by RIA. The dose–response (bound/total ratio for labeled hormone or B/T) curve for the labeled hormone tested is compared to the standard RIA dose–response curve. In this application, the relative potency is identical with the specific activity in terms of radioactive counts per picogram of LHRH. Unconstrained curve fitting for the labeled ligand resulted in a physically impossible negative estimate of *d*. This curve did not extend sufficiently into the high-dose region because total radioactivity is limited in practice to approximately 10^6 cpm per tube. Constraining both curves to share identical values of *a* and *d* (Fig. 1*B*) (as would be expected for a valid RIA system, assuming that nonspecific counts have been measured correctly) results in a more suitable fit. The specific activity of ^{125}I-labeled LHRH was estimated as 5,344 ± 3,584 counts/pg when analyzing each curve separately, and as 3,251 ± 220 counts/pg when using constraints. Thus, use of constraints has resulted in a dramatic tenfold reduction in the size of the confidence limits, when potency is calculated as the ratio of the ED_{50}'s. The curves may also be constrained to share a common slope *b* with no significant decrease in the goodness of fit (Table 1).

2) Radioreceptor assays for neurotransmitters. In radioligand assays for hormones and neurotransmitters (acetylcholine, catecholamines, opiates, etc.), it is customary to construct dose–response curves for many analogues. Not uncommonly, these fall into groups of agonists and antagonists with similar properties, readily apparent by visual inspection of the dose–response curves. Here, we wish to characterize the ED_{50} or ID_{50} of each agent, its slope factor *b* and to quantitatively and objectively identify "families" of agents with identical *b* values (parallel curves). Figure 2 shows competitive binding curves for the labeled dopaminergic agonist dihydroergocryptine ([^{3}H]DHEC) in the presence of increasing amounts of unlabeled antagonists (Fig. 2*A*) and agonists (Fig. 2*B*). The curves for the antagonists (Fig. 2*A*) can be constrained to be parallel (all *b*'s equal) without any significant effect on the goodness of fit (Table 2); further, the common slope factor (*b*) can be set equal to a value of 1 without deleterious effect (Table 2).

In contrast, each of the four curves for agonists (Fig. 2*B*) has a slope factor (*b*) significantly lower than unity: 0.68 ± 0.06, 0.45 ± 0.04, 0.40 ± 0.03, and 0.40 ± 0.03. We infer that the curves for the agonists are not parallel because the additional constraint of parallelism results in a deterioration of goodness of fit, with a significant increase in the average scatter around the curves (increased *F* test value) and a significant nonrandomness of the residual signs (Table 3). This lack of parallelism for the agonists is mainly due to the first curve (apomorphine), which is steeper than the other three curves.

TABLE 1. *Statistical analysis for goodness of fit for various models for Fig. 1*

Parameters Shared	*F* Test	Sign Runs	
		Curve 1 (7, 6)	*Curve 2* (7, 6)
None	1.00	5	4
a, *d*	0.79	4	4
a, *b*, *d*	1.06	5	5
b, *d*, and *a* = 0	0.97	5	4

The parameters shared between the two curves are indicated in the first column. The *F* test for the effect of the constraints on the residuals is indicated in column two. The number of sign runs of the residuals for each curve is indicated in columns three and four. The total number of observations is 7 and 6 for native LHRH (*curve 1*) and labeled hormone (*curve 2*), respectively. For each curve, the expected range ($P > 0.05$) of the sign runs is indicated in parentheses. Fig. 1, *A* and *B* correspond to lines 1 and 4 of this table. Based on these statistical tests, we infer that both curves share common values for *b* and *d*, and *a* = 0.

[7] The potency estimate of a test substance relative to a standard drug is the ratio of equipotent doses of the test and the reference substance. There has been considerable debate on the applicability of the relative potency estimate method to nonparallel curves (8, 9, 16). In the case of nonparallelism, the horizontal distance between the two curves on a logarithmic scale and hence the potency estimate is variable. For example, the potency estimate based on the ratio of doses producing half-maximal response (ED_{50}) would differ from those based on ED_{20} or ED_{80}.

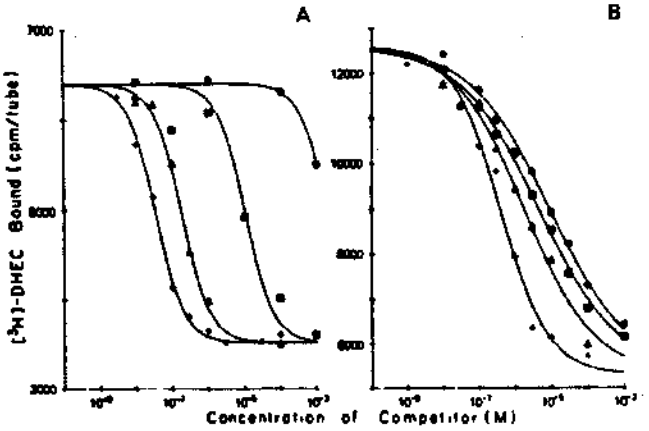

FIG. 2. Displacement of tritiated dihydroergocryptine [³H]DHEC from bovine anterior pituitary membrane receptors *A*: by the antagonists haloperidol (+), chlorpromazine (▲), phentolamine (■) and propanolol (●) or *B*: by the agonists apomorphine (+), dopamine (▲), (−)epinephrine (■) and (−)norepinephrine (●). Solid lines represent fitted curves subject to following constraints: (*A*), common a, b, d for all curves, and (*B*), common a, d for all curves, common b for epinephrine and norepinephrine. Data of Marc Caron, ref. 6.

TABLE 2. *Tests for goodness of fit for various models for Fig. 2A*

Parameters Shared	*F* Test	Run Tests for Curve			
		1 (4, 10)	*2* (3, 9)	*3* (2, 6)	*4* (2, 6)
a, d	1.00	7	6	5	4
a, b, d	1.57	7	6	5	3
a, d, and $b = 1$	1.39	7	6	4	3

Simultaneous unconstrained curve fitting did not converge; therefore the least constrained case (a and d shared for all curves) was used as a basis for the *F* tests in column two. The total number of observations were 12, 11, 7, 7 for haloperidol (*curve 1*), chlorpromazine (*curve 2*), phentolamine (*curve 3*), and propanolol (*curve 4*), respectively. For each curve, the expected range ($P > 0.05$) of values of the number of sign runs is indicated in parentheses. The curves shown in Fig. 2*A* correspond to line two of this table. The tests shown were nonsignificant ($P > 0.05$); therefore, one may infer that all curves have a common a, d, and b, and that $b = 1$.

TABLE 3. *Tests for goodness of fit for various models for Fig. 2B*

Parameters Shared	*F* Test	Run Tests for Curves			
		1 (3, 9)	*2* (3, 9)	*3* (3, 9)	*4* (3, 9)
None	1.00	5	7	5	6
All a, d	2.19	4	7	5	5
All a, d, and $b_3 = b^4$	1.94	4	7	5	5
All a, d, and $b_2 = b_3 = b_4$	2.16	4	5	5	3*
All a, b, d	6.88*	3*	7	5	3*

The number of observations was 10 for apomorphine (*curve 1*), dopamine (*curve 2*), epinephrine (*curve 3*), and norepinephrine (*curve 4*). When all a's, b's, and d's are set equal, the *F* test is highly significant ($P < 0.005$), and some run tests (indicated by an asterisk) reach the level of significance ($P = 0.05$). Thus, we reject the hypothesis that all curves are parallel and share the same limits a and d.

These latter curves may be constrained to be parallel (same b). Consideration of the nonparallelism of displacement curves for agonists and antagonists may lead to new insights into the mechanisms of interaction of these agents with their specific receptor(s) and permit

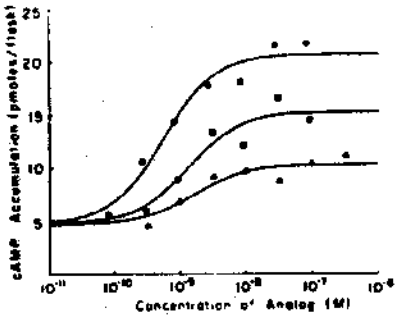

FIG. 3. Dose-response curves for human chorionic gonadotropin (hCG, ●) and partial agonists NG-hCG (■), NGAm-hCG (▲), on cyclic adenosine monophosphate (cAMP) production by rat Leydig cells. Solid lines represent curves fitted with shared a and b. Data of William Moyle, ref. 25.

classification of compounds.

3) Biossay of partial agonists. In the bioassay of a family of agonists and partial agonists, the basal response level (a) may remain the same but the maximal response (d) will be smaller for the partial agonists. However, the curves may reveal the same "steepness" (same b). Appropriate evaluation of the potency estimate of the partial agonists can best be obtained by simultaneous constrained curve fitting. Figure 3 shows Leydig cell adenosine 3',5'-cyclic monophosphate accumulation in response to varying doses of hCG and two related partial agonists. The curves have been forced to share a common a and b. The additional constraint of a common c (ED_{50}) does not significantly alter the goodness of fit of the curves. Thus, these three curves would be nearly superimposable if their responses were normalized to 100% of their respective maximum.

4) DNA-RNA hybridization. Whereas parameters b and c usually reflect intrinsic properties of the system, parameters a and d often vary between experiments, depending on experimental conditions. Simultaneous curve fitting may be used as an elegant and efficient method for pooling information from several experiments while minimizing problems of between-experiment variability in some parameters. Figure 4 shows DNA-RNA hybridization curves for ovalbumin mRNA purified from chick oviduct and total mRNA from chick oviducts treated with estrogen. Here the RNA samples were repeatedly assayed in different experiments with varying d values (maximum binding capacity). The d

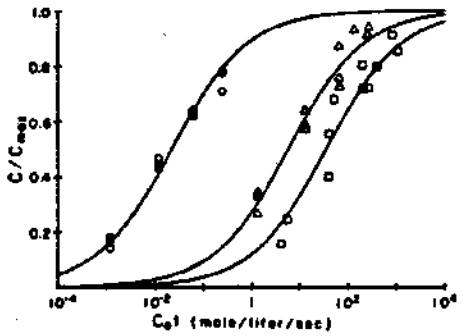

FIG. 4. Hybridization to cDNA of purified mRNA for ovalbumin (○), and of total mRNA from chicks treated with estrogens for 4 (△) or 18 days (□). Each mRNA preparation was assayed in 3 different experiments. For curve fitting, parameter a was set at zero and b was common to all curves. Parameter c was shared between individual curves for same mRNA preparation. Parameter d (extrapolated maximum percentage of initial mRNA associated at infinite time and concentration) varies between experiments (82 ± 7, 58 ± 4, 56 ± 4, 47 ± 4) and was pooled only for curves within the same experiment. Resulting curves are shown normalized to their respective d. Data of S. E. Harris, ref. 19.

TABLE 4. *Analysis of goodness of fit for various models for Fig. 4*

Parameters Shared	F Test	Confidence Level
$a = 0$	1.00	
$a = 0$; and c for a given preparation	1.79	$P = 0.25$ (NS)
$a = 0$, b, d, and c for a given preparation	8.58*	$P < 0.005$
$a = 0$; b, c for a given preparation, and d within the same experiment	2.18	$P = 0.1$ (NS)

Each mRNA preparation was assayed once in three different experiments for a total of nine curves. Use of constraints was intended for pooling curves from the different experiments. The F tests for some representative cases are shown together with their approximate confidence levels. The sign run tests were not contributory because of the small number of observations (four) per individual curve. The curves shown in Fig. 4 correspond to line four of this table. The asterisk indicates statistical significance.

values were pooled for all curves within the same experiment while constraining the c values ($C_0t_{1/2}$) to be equal for all curves for the same substance. The additional constraint of parallelism (common b) did not alter the goodness of fit. Forcing all the d values to be equal in addition to the constraints for b and c resulted in significant degradation of the goodness of fit (Table 4). In Fig. 4, the data from each of the nine original curves are shown normalized to 100% of the d values for their corresponding experiment.

DISCUSSION

The four-parameter logistic equation seems to appropriately describe, within experimental accuracy, most *symmetrical sigmoidal dose-response curves. In those* cases in which sigmoidal curves are significantly asymmetrical, the logistic model could be extended by incorporating one or two additional asymmetry parameters (26). For a complex titration curve involving multiple classes of binding sites with widely disparate ED_{50}'s, one may use a summation of logistic terms. This has been applied to DNA-RNA hybridization data (23).

The present program may be used to calculate "parallel line potency estimate" for in vivo bioassays, without need for truncation to a central linear segment or use of logit transform of the response (17). Truncation of the response curves results in systematic loss of information by neglecting the end parts of the curves, whereas the popular use of the logit–log linear regression relies on independent estimates of the limiting values a and d, which cannot be readjusted during the fitting process. Simultaneous curve fitting with the four-parameter logistic model uses the available experimental data most efficiently and allows for a greater flexibility in adjusting to varying experimental conditions.

Waud (37-39) has pioneered the use of computer analysis of families of dose–response curves. He has applied simultaneous curve fitting based on a three-parameter logistic equation for estimating dissociation constants of agonists and antagonists assayed by pharmacological "null" methods. The computer programs that he developed are most appropriate for that specific purpose. The data analysis that we describe, being more general, may not be as efficient in such specialized cases because we do not specify any underlying relationship between the ED_{50}'s of the curves as for "null" methods applied to the case of competitive antagonists.

The four-parameter logistic equation often represents a significant improvement over the three-parameter version because the base-line level (or the background or the nonspecific level) is included among the parameters instead of being considered as a perfectly known constant (20, 29). Provision for weighting may be essential when the range of observed responses is quite large, resulting in unavoidable nonuniformity of variance of the response metameter (30). Flexibility in the choice of the shared parameters and multiple statistical tests for goodness of fit constitute the major advances of the program described here.

The use of constrained simultaneous curve fitting for testing the equality of parameters is preferable to testing the identity of parameters estimated from curves fitted individually. The standard errors and confidence limits of parameter estimates in nonlinear regression are only approximate, and any conclusion regarding the equality of corresponding parameters is only approximate. In contrast, simultaneous constrained curve fitting permits testing for equality of parameters by inspecting the consequences of forcing them to be equal.

Most investigators still use simple graphic methods and subjective visual curve fitting. Perhaps this has been justifiable: computerized curve fitting of one curve at a time may fail to converge on correct values or even converge at all. An experienced experimentalist will automatically employ constraints (forcing the curves to *assume desired characteristics based on an underlying* model or previous results). Now, the present computer program should retain the advantages of the subjective methods, but also provide objective estimates of the reliability of the parameters.

In conclusion, we have described a simple computerized method for efficient data analysis of families of

dose–response curves. The method proved to be extremely versatile and generally applicable to many different types of bioassays and binding assays, and other physiological or pharmacological dose–response curves. The program (ALLFIT) is readily adaptable to larger desk-top computers and is available on request.

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